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RESEARCH ARTICLE

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Characterisation and expression of four mRNA sequences encoding glutathione S-transferases pi, mu, omega and sigma classes in the Pacific oyster *Crassostrea gigas* exposed to hydrocarbons and pesticides

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Abstract Hydrocarbon and pesticide pollution in coastal ecosystems can disturb marine bivalve metabolism. In this study, we characterised four full-length cDNA sequences encoding glutathione S-transferases (GSTs) in the Pacific oyster *Crassostrea gigas*. A BLAST X search showed that these four sequences encode GSTs from four different classes: GST pi, sigma, mu and omega. A phylogenetic analysis of GST was made to determine the position of oyster GST compared to invertebrate and vertebrate sequences. We developed a semi-quantitative, multiplex RT-PCR to follow the expression of these four GSTs in tissues of oysters exposed to hydrocarbons and two pesticide treatments (glyphosate and a mixture composed of atrazine, diuron and isoproturon) under experimental conditions. Our results showed strong differential expression of these four GSTs that was both tissue specific as well as time and treatment dependent. We observed that expression levels were higher in digestive gland than in gill tissues in pesticide-exposed oysters. Furthermore, omega and mu class GST mRNA expression in the digestive gland might be useful as a possible marker of hydrocarbon exposure, while pi and sigma class GST mRNA expression in the digestive gland may be similarly useful as a marker of pesticide exposure in monitoring programmes.

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Introduction

Xenobiotics biotransformation processes in eukaryotic cells can be categorised by phase. Phase I is characterised by the oxygenation of xenobiotics and endogenous substrates by the inducible cytochrome p450–dependent microsomal monooxygenase. Reduction of lipophilic compounds by Cyp450 results either in directly excreted polar metabolites or in more reactive molecules, which are used as substrates by phase II enzymes (Lüdeking and Köhler 2002). Phase II enzymes catalyse the conjugation of the xenobiotic to the endogenous compounds. Glutathione S-transferases (GSTs) are the major phase II enzymes that conjugate glutathione and electrophilic substrates (Lüdeking and Köhler 2002; Van der Oost et al. 2003). A third phase has also been characterised; enzymes involved in this phase are members of the multi-drug resistance (MDR) and multi-xenobiotic resistance (MXR) protein families (Lüdeking and Köhler 2002). These proteins, first described in cancer cell lines (Endicott and Ling 1989) and in marine invertebrates (McFadzen et al. 2000), act as a pump involved in the export of xenobiotics out of the cell (Lüdeking and Köhler 2002).

Glutathione S-transferases are comprised of classes of dimeric enzymatic proteins that catalyse the conjugation of glutathione to a wide variety of hydrophobic compounds through the formation of a thioether bond with their electrophilic centre (Hayes and Pulford 1995). Based on amino acid sequence identity, enzymatic properties and immunological reactivity, there are at least eight major classes of GSTs, designated alpha, kappa, mu, pi, sigma, omega, theta and zeta (Hayes and Pulford 1995; Pemble et al. 1996; Board et al. 1997; Sheehan et al. 2001). These enzymes have evolved as a cellular protection system against a range of xenobiotics and oxidative metabolic by-products, and, in particular, are known to metabolise a number of environmental carcinogens. The wide range of GST isoforms present in

Table 1 *Crassostrea gigas*. Combinations of primers used in the amplification of the 5′ and 3′ UTRs of the cDNA encoding the four GSTs

Genes	Primer sequences
GST mu	sense GGGCTTGGCCAGCCAATCAGATTGCTGCT
	antisense TCTGATTGGCTGGCCAAGCCCTC
GST pi	sense GAGGCGCGTCCGAAGCTAGCGGC
	antisense TTTCCATGAAAGGCCAATCTTCC
GST omega	sense TGGCCATGGTTTGAACGTATTCT
	antisense TCGGGATACACCTGGTCCAAATA
GST sigma	sense AACCTGAGTGAATACCTCTCCTCCAGACC
	antisense CCAGCTAAACCGAACTCCCTGGCCAAGTA

the various classes provides cells with an efficient way of scavenging the huge number of potentially toxic compounds encountered. They are ubiquitous enzymes reported in most animal phyla, e.g. molluscs (Fitzpatrick and Sheehan 1993; Fitzpatrick et al. 1995; Blanchette and Shingh 1999, Vidal et al. 2002), annelids (Stenersen et al. 1979), crustaceans (Keeran and Lee 1987; LeBlanc and Cochrane 1987) and mammals (Rouimi et al. 1996). Numerous studies with molluscs have shown that GST enzyme activity is either inducible or unchanged by exposure to various xenobiotics (Khessiba et al. 2001; Alves et al. 2002; Cheung et al. 2002; Gowlan et al. 2002; Petushok et al. 2002; Torres et al. 2002; Le Pennec and Le Pennec 2003). To our knowledge, little information is available on GST cDNA, gene sequences, or mRNA expression in molluscs.

In this study, we characterised, for the first time, the complete cDNA sequences of four GSTs in the marine bivalve *Crassostrea gigas*, each belonging to a different class. The mRNA expression of the pi, mu, omega and sigma class GSTs and their potential use as biomarkers of contaminant exposure were investigated. We developed a semi-quantitative, multiplex RT-PCR (real-time polymerase chain reaction) method to analyse GST mRNA expression in oysters exposed to hydrocarbons and two pesticide treatments. One pesticide exposure, designated ADI, was to a mixture of 2-chlor-4-ethylamino-6-isopropylamino-1,3,5,-triazin (atrazine), 3-(3,4-dichlorphenyl)-1–1-dimethyl-harnstoff (diuron) and 3-(4-isopropylphenyl)-1,1-dimethylharnstoff (isoproturon) and the second was to *N*-(phosphonomethyl)glycine (glyphosate).

Materials and methods

Experimental design

Adult oysters (10–11 cm, *Crassostrea gigas*) were collected from La Pointe du Château (Brittany, France) in December 2001 for the hydrocarbon experiment and in September 2002 for the pesticide experiments. After an acclimatisation period of 7 days in aerated 0.22-μm-filtered seawater at constant temperature and salinity (15°C and 34‰, respectively), oysters were challenged as follows. Groups of 20 oysters were exposed for 3 weeks to a 0.1% mixture of hydrocarbon, consisting of the water-soluble fraction of domestic fuel homogenised for 3 days in filtered seawater (Snyder et al. 2001). Other groups of 20 oysters each were exposed for 4 weeks to either a mixture of three herbicides (2 μg atrazine l^{-1}; 0.5 μg diuron l^{-1} and 1 μg isoproturon l^{-1}, mixture called ADI) or to 2 μg glyphosate l^{-1}. Another group of 20 oysters was maintained in seawater, without contaminant, as a control. No mortality was observed in the control or treated oysters.

Extraction of total RNA and cDNA synthesis (reverse transcription)

Total RNA was extracted from the digestive glands of treated oysters after 0, 7, 15 and 21 days of exposure to the hydrocarbon mixture, and from the digestive glands and gills of treated oysters after 0, 7, 15, 21 and 30 days of exposure to the pesticide treatments, according to the method based on extraction in guanidium isothiocyanate (Strohman et al. 1977). Matching extractions from control oysters were done for each treatment. For each sample, 10 μg of RNA was submitted to reverse transcription using oligo dT anchor primer (GAC CAC GCG TAT CGA TGT CGA $CT_{(16)}$V) and M-MLV reverse transcriptase (Promega).

Cloning and sequencing of 5′- and 3′-flanking regions of omega, pi, mu and sigma GST cDNA

Total RNA was extracted from the digestive gland of one 21-day hydrocarbon-exposed oyster for 5′- and 3′-UTR amplification of GSTs mu, pi and omega and from the digestive gland of one control oyster for 5′- and 3′-UTR amplification of GST sigma. The RT-PCR was carried out according to the procedure described above. The procedures for the generation of 5′- and 3′-untranslated regions (UTR) GST cDNA were carried out according to the commercial protocol 5′/3′ rapid amplification of cDNA ends (5′/3′ RACE Kit, Roche) using specific primers designed from the sequences obtained in suppressive subtraction hybridisation (SSH) libraries (Boutet et al. 2004) and reported in Table 1. The 3′ UTR was amplified as follows: 200 ng of reverse transcription product and 2 mM $MgCl_2$; 10 pmol each of PCR anchor primer (GAC CAC GCG TAT CGA TGT CGA C) and specific primer were submitted to amplification using one cycle at 94°C for 2 min, 58°C for

2 min and 72°C for 1 min 30 s; then 40 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 1 min; with a final step at 72°C for 10 min. Amplification of the 5′ UTR was carried out according to the following procedure: 200 ng of reverse transcription product was treated with terminal deoxynucleotidyl transferase (Promega) and dATP to generate a polyA tail at the 5′-end. Subsequently, the products were submitted to denaturation at 94°C for 2 min; then 10 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 1 min; followed by 30 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 1 min, with an increase of 20 s per cycle for the elongation time; and a final step at 72°C for 15 min, with 2 mM $MgCl_2$ and 10 pmol each of oligo dT anchor primer and specific antisense primer (Table 1). The resulting cDNA fragments corresponding to the 5′ and 3′ UTRs were cloned into pGEM-T vector (Promega) and sequenced using a Li-COR IR2 (Sciencetech) and Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Bioscience).

Semi-quantitative, multiplex RT-PCR

To perform semi-quantitative RT-PCR, the total amount of isolated total RNA was measured by UV-spectroscopy at 260 nm. PCR experiments in which each primer pair was omitted in the primer mix showed that the amplification of genes investigated in the PCR was not altered by the presence of the other primer pairs. Amplification was performed as follows: one cycle at 94°C for 2 min, 55°C for 1 min and 72°C for 1 min 30 s; 35 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min; followed by a final 10 min extension at 72°C. PCR products were separated on 1.5% agarose gels using TBE-buffer and photographed after ethidium bromide staining. The primer pairs used for amplification (10 pmol each) and the length of the generated fragments are reported in Table 2. A PCR amplification control (28S ribosomal DNA) was used for all experiments and was amplified as described above with the primers sense AAG GGC AGG AAA AGA AAC TAA C and antisense TTT CCC TCT AAG TGG TTT CAC. Quantification of band intensities was measured using Gene Profiler 4.03 software (Scanalytics), and ratios between OD of bands corresponding to GSTs and 28S were calculated.

Molecular phylogenetic analysis

Analyses were performed on the four GST cDNA sequences from various species, including vertebrates and invertebrates. Amino acid sequences were aligned with CLUSTAL X software (Thompson et al. 1997). Molecular phylogenetic trees were constructed using the neighbour-joining (NJ) algorithm in the PHYLIP software and the phylogenetic package MEGA2 (Kumar et al. 2001). Amino acid differences between sequences were corrected for multiple substitutions using a gamma correction. In this correction, α, the shape parameter of the gamma distribution, was set to 2. With $\alpha = 2$, the distance between any two amino sequences, d_{ij}, is approximately equal to Dayhoff's (1979) PAM distance per site (Kumar et al. 1994). Phylogenetic trees were also generated using parsimony. For this analysis, amino acid changes were unweighted; thus; a change from one amino acid to any other was equally probable. Support for the major nodes within both distance and parsimony trees were evaluated by bootstrapping the data; 1000 bootstrap replicates of the whole data set were examined (Felsenstein 1995).

Results

Identification of four mRNA sequences encoding GSTs in *Crassostrea gigas*

Sequence data was submitted to GenBank: AJ557140 1124 bp *C. gigas* GST pi mRNA, complete coding sequence (cds); AJ557141 908 bp *C. gigas* GST omega mRNA, complete cds; AJ558252 894 bp *C. gigas* GST mu mRNA, complete cds; and AJ577235 1002 bp *C. gigas* GST sigma mRNA, complete cds.

Initially, partial sequences of the four GSTs were obtained from hydrocarbon-exposed *C. gigas* digestive gland SSH libraries (Boutet et al. 2004): 384, 444, 291 and 573 bp encoding pi, omega, mu and sigma class GSTs, respectively. Specific primers were designed to amplify the 5′ and 3′ UTRs of each GST cDNA. The resulting sequences contained open reading frames of 738 bp (245 amino acids) for the pi class GST (Fig. 1), 732 bp (243 amino acids) for the omega class GST (Fig. 2), 648 bp (215 amino acids) for the mu class GST

Table 2 *Crassostrea gigas*. Combinations of primers used in semi-quantitative, multiplex RT-PCR and length of the generated fragments

Genes	Primer sequences	Length of generated fragments (bp)
GST mu	sense ATGTCGACGCTTGGCTACTGGAACATTAG antisense TTGAACAATGCAAACTTGTTGTTGACGGG	200
GST pi	sense ATGGCGGACTGGGAAATTCTTTACCACAA antisense GCTGTTGGTGTCCTGTGGGTGTTTGGGTA	690
GST omega	sense TATTTGGACCAGGTGTATCCCGA antisense AGAATACGTTCAAACCATGGCCA	280
GST sigma	sense TACTTGGCCAGGGAGTTCGGTTTAGCTGG antisense GGTCTGGAGGAGAGGTATTCACTCAGGTT	390

Fig. 1 *Crassostrea gigas*. The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase (GST) class pi in oyster (AJ557140). Stop codon is marked by an *asterisk* and untranslated regions are in *lower case letters*. Polyadenylation signals are *underlined* and messenger stability-determining motifs ATTTA and ATTTG are *enclosed in boxes*

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gacctgaacagtcatacatcatatatataggacttcaaacatttgaaagagtgttgacttttgtttaaacag      72

                                                           M   A   D   W   E       5
aagaacggaatttatcatcgtgcaaacagcgaggttttagttacacacagaa ATG GCG GAC TGG GAA     139

 I   L   Y   H   N   I   P   C   A   G   R   A   E   F   V   R   L   I        23
ATT CTT TAC CAC AAC ATA CCG TGT GCT GGA AGA GCT GAA TTT GTT CGT TTG ATC      193

 F   E   E   A   G   V   P   Y   T   E   P   M   K   T   Q   E   E   I        41
TTC GAA GAA GCT GGG GTT CCT TAT ACA GAA CCA ATG AAA ACC CAA GAG GAA ATC      247

 R   D   T   I   M   N   N   K   L   G   G   F   P   V   M   F   P   P        59
CGA GAT ACG ATC ATG AAC AAT AAA CTC GGA GGT TTT CCG GTC ATG TTC CCT CCT      301

 V   L   K   R   G   D   F   H   L   C   Q   T   S   V   I   C   K   Y        77
GTC TTG AAA CGA GGC GAT TTT CAC CTC TGT CAG ACG TCA GTG ATA TGT AAG TAC      355

 L   G   E   Q   F   R   L   M   P   K   S   E   E   E   K   W   Q   A        95
CTG GGG GAA CAA TTT AGA CTG ATG CCA AAA TCA GAA GAG GAA AAA TGG CAG GCG      409

 D   Q   V   N   A   T   I   H   D   F   V   A   E   G   R   L   E   S       113
GAT CAA GTT AAC GCC ACC ATT CAC GAC TTT GTG GCA GAA GGA AGA TTG GAA TCC      463

 R   G   A   K   S   I   N   Y   Y   F   V   G   R   L   A   F   H   G       131
CGC GGC GCT AAA AGT ATC AAC TAT TAT TTC GTA GGA AGA TTG GCC TTT CAT GGA      517

 K   H   W   V   G   S   Y   H   D   Q   K   E   E   T   Q   P   Y   I       149
AAG CAT TGG GTG GGG TCT TAC CAC GAC CAA AAG GAA GAA ACA CAG CCG TAT ATT      571

 D   W   F   V   K   E   R   L   P   K   W   L   K   H   F   E   L   V       167
GAT TGG TTT GTC AAA GAG AGG CTA CCG AAA TGG CTG AAG CAT TTT GAG TTA GTT      625

 L   K   N   N   N   G   G   N   G   F   C   F   G   E   E   V   T   Y       185
CTG AAA AAC AAC AAT GGC GGA AAC GGT TTC TGC TTT GGA GAG GAA GTG ACG TAT      679

 V   D   L   A   L   L   Q   C   L   R   G   C   E   A   S   Y   K   K       203
GTC GAC TTG GCA CTA CTC CAG TGC CTC CGC GGT TGT GAA GCG TCT TAT AAA AAG      733

 G   F   E   S   A   D   Y   C   P   S   L   K   A   F   K   A   Q   M       221
GGT TTC GAG TCG GCA GAT TAT TGC CCT TCC CTA AAG GCG TTC AAA GCC CAG ATG      787

 E   A   R   P   K   L   A   A   Y   Y   K   S   E   R   Y   P   N   T       239
GAG GCG CGT CCG AAG CTA GCG GCC TAT TAC AAG TCA GAG CGG TAC CCA AAC ACC      841

 H   R   T   P   T   A   *                                                   245
CAC AGG ACA CCA ACA GCA TGA tgtgacgtggacgacaaaaaatatgacgtcatcataattgagaa     906
tttcagcaattgcttgtttttgttgttgttgttgtgaagtaaaacttttctgtgtagttgcttatttattt      978
gaagaattctcttgcgcttattttgctttaaaagattgatcttattgacttcattaataaatacatctattta   1050
tttggatataagatataatatatgtatatgtttatgtagaaataaacaacaaaatttgaaaaaaaaaaaaaa    1122
aa                                                                          1124
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(Fig. 3) and 609 bp (202 amino acids) for the sigma class GST (Fig. 4). We also observed multiple ATTTA(G) motifs in the untranslated regions of the four GSTs correlated with transcript stability. The cDNA encoding the mu class GST had one ATTTG motif in the 3′ UTR. The omega class GST had two ATTTG motifs and one ATTTA motif in the 3′ UTR. The pi class GST had one ATTTA and one ATTTG motif in the 5′ UTR and two ATTTG motifs and one ATTTA motif in the 3′ UTR. And, the sigma class GST had two ATTTA motifs in the 3′ UTR. Moreover, the pi class GST cDNA contained two polyadenylation signals in its 3′ UTR. The four GST cDNAs encode proteins with a calculated molecular masses of 28.4 kDa (pi), 28 kDa (omega), 25.1 kDa (mu) and 23.6 kDa (sigma) (MWCALC software, http://www.infobiogen.fr).

A phylogenic tree was constructed by analysing the amino acid sequences of *C. gigas* GST pi, mu, sigma and omega and the GST sequences of different invertebrate and vertebrate species (Fig. 5). *C. gigas* GSTS mu, omega and sigma clustered with the corresponding GST classes of other species and were closer to invertebrate

Fig. 2 *Crassostrea gigas*. The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class omega in oyster (AJ557141). Stop codon is marked by an *asterisk* and untranslated regions are in *lower case letters*. Polyadenylation signals are *underlined* and messenger stability-determining motifs ATTTA and ATTTG are *enclosed in boxes*

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                              M   P   T   Q   Q   S   F   A   T   G   S   A      12
agagagaaattattgtaaaaacca ATG CCG ACC CAA CAA TCA TTT GCT ACA GGT TCC GCC      60

 C   P   E   L   E   A   G   T   L   R   V   Y   S   M   R   F   C   P         30
TGT CCG GAA TTG GAG GCA GGG ACT CTC CGA GTA TAC AGC ATG AGG TTC TGT CCG       114

 Y   A   Q   R   A   L   L   V   L   T   Y   K   N   I   P   H   E   V         48
TAT GCT CAA CGA GCC CTG CTG GTC CTG ACA TAC AAA AAT ATA CCA CAT GAA GTG       168

 V   N   I   N   L   K   N   K   P   E   W   F   L   Q   K   N   P   L         66
GTC AAC ATC AAT CTG AAA AAT AAA CCG GAA TGG TTT CTG CAG AAG AAC CCA CTG       222

 G   R   V   P   T   L   E   K   D   D   R   I   V   Y   E   S   A   I         84
GGG CGG GTT CCC ACC TTA GAG AAA GAT GAC AGA ATC GTG TAC GAG TCC GCT ATC       276

 C   C   D   Y   L   D   Q   V   Y   P   D   N   K   L   T   P   D   D        102
TGC TGT GAC TAT TTG GAC CAG GTG TAT CCC GAT AAC AAG CTG ACC CCA GAT GAC       330

 P   Y   R   Q   A   R   D   K   M   T   V   E   V   F   S   Q   F   V        120
CCT TAC CGT CAG GCC CGG GAC AAG ATG ACC GTG GAG GTC TTC TCT CAG TTT GTT       384

 S   D   F   Q   K   M   M   S   S   P   P   Q   E   K   P   E   S   L        138
TCG GAT TTT CAA AAA ATG ATG AGT TCA CCA CCG CAA GAG AAA CCC GAG AGT TTA       438

 Q   K   I   K   N   N   L   C   E   F   E   S   S   L   T   A   R   Q        156
CAA AAG ATC AAA AAC AAC TTA TGT GAG TTT GAG AGC AGC CTA ACA GCA AGG CAA       492

 G   A   Y   F   G   G   N   A   V   Q   M   L   D   F   L   L   W   P        174
GGC GCC TAC TTT GGA GGG AAC GCA GTG CAG ATG CTA GAC TTC CTG CTG TGG CCA       546

 W   F   E   R   I   L   I   F   A   K   V   V   P   L   T   F   S   L        192
TGG TTT GAA CGT ATT CTC ATC TTC GCA AAA GTT GTT CCG CTG ACG TTC TCT TTA       600

 E   D   Y   P   A   L   C   E   W   T   K   K   M   P   E   C   P   A        210
GAG GAC TAT CCA GCT TTG TGT GAA TGG ACA AAG AAA ATG CCG GAA TGT CCG GCC       654

 V   Q   K   C   R   L   D   P   Q   Q   F   L   E   F   Y   K   S   T        228
GTC CAA AAA TGT CGA TTG GAC CCT CAG CAG TTT TTG GAA TTT TAC AAA AGT ACA       708

 K   A   G   A   P   D   Y   D   V   P   K   T   S   Q   S   *                244
AAA GCT GGC GCA CCT GAT TAT GAC GTA CCT AAA ACT TCA CAG TCA TAA acgaagag      764
aaagtgaacaacttggtactaacatgtatatacattttatgttctgtaacacaatatttggttttacaatt       836
tctgtttcaaaattattttcttccgctgtatttgatttctcaaataaattaatttaaaataaaaaaaaaaaa      908
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species than vertebrates, as expected. Interestingly, *C. gigas* GSTs constitute distinct branches from other non-mollusc species. The *C. gigas* GST pi does not clearly cluster with the GST pi of other species, despite the fact that BLAST P analysis showed high similarity with other GST pi sequences.

Glutathione S-transferase expression analysis using semi-quantitative, multiplex RT-PCR

Based on the sequencing information obtained, a semi-quantitative, multiplex RT-PCR was designed to simultaneously assay the differential expression of the four GST genes in *C. gigas*. The results of semi-quantitative, multiplex RT-PCR showed a strong differential expression between the different classes of GSTs, tissues and treatments. Analysis of GST expression in the digestive gland of oysters exposed to hydrocarbons displayed a clear induction of the GST mu mRNA after 21 days of exposure, the GST omega mRNA (16-fold compared to the control after 14 and 21 days) and the GST pi (up to 13-fold) after 21 days of exposure. A weak decrease of GST sigma mRNA (twofold compared to the control) was also observed after 21 days of exposure (Fig. 6). Regarding Fig. 6, we observed that the omega class GST mRNA was more abundant in oyster digestive gland than were the other GST classes. Analysis of GST expression in the gill of oysters showed similar patterns of expression as in the digestive gland, but with a higher level of expression for most of the GSTs. No significant variations in GST expression level were observed in control oysters sampled at 7, 14 and 21 days during the hydrocarbon experiments,

Fig. 3 *Crassostrea gigas*. The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class mu in oyster (AJ558252). Stop codon is marked by an *asterisk* and untranslated regions are in *lower case letters*. Polyadenylation signals are *underlined* and messenger stability-determining motifs ATTTA and ATTTG are *enclosed in a box*

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                                                              M   S   T      3
gggagtcgctggtgcgtgcacatgctagttcgattcgctgcactttttttcatcaagcaaa ATG TCG ACG   69

 L   G   Y   W   N   I   R   G   L   G   Q   P   I   R   L   L   L   N     21
CTT GGC TAC TGG AAC ATT AGA GGG CTT GGC CAG CCA ATC AGA TTG CTG CTG AAC   123

 Y   V   G   E   E   F   D   D   V   Q   Y   E   L   G   D   A   P   D     39
TAT GTC GGA GAG GAA TTC GAT GAT GTA CAG TAT GAA CTT GGA GAT GCA CCC GAC   177

 Y   S   R   E   E   W   L   S   V   K   N   T   L   G   L   A   F   P     57
TAT AGC AGA GAA GAA TGG CTC TCT GTC AAA AAC ACT CTA GGA CTA GCC TTC CCA   231

 N   I   P   Y   Y   I   D   D   D   I   K   I   T   Q   S   N   S   I     75
AAT ATT CCC TAT TAC ATT GAT GAT GAT ATA AAA ATT ACA CAA AGT AAC TCC ATA   285

 L   R   Y   I   G   D   K   H   G   L   L   G   K   T   P   R   D   K     93
TTG AGG TAT ATT GGA GAT AAA CAT GGC CTG TTA GGA AAA ACT CCC CGA GAC AAA   339

 V   D   C   D   M   M   V   E   N   A   M   D   F   R   N   G   V   I    111
GTG GAC TGT GAT ATG ATG GTG GAG AAC GCC ATG GAT TTT AGA AAT GGG GTC ATT   393

 R   L   C   Y   D   N   D   Y   E   K   I   K   D   D   Y   F   A   N    129
CGG TTG TGC TAC GAC AAC GAC TAC GAA AAG ATC AAG GAC GAC TAC TTT GCC AAT   447

 V   K   D   K   L   R   Q   F   D   T   F   L   G   D   K   P   W   F    147
GTC AAG GAC AAA CTA AGA CAG TTT GAC ACG TTC CTT GGA GAC AAA CCT TGG TTC   501

 A   G   D   G   I   T   I   C   D   F   P   L   Y   E   L   L   D   Q    165
GCT GGA GAT GGT ATC ACC ATC TGT GAC TTC CCA TTG TAC GAG TTA CTA GAC CAG   555

 H   R   L   M   K   P   G   I   L   D   D   Y   P   N   L   T   K   F    183
CAC AGA CTG ATG AAG CCT GGG ATA CTA GAC GAT TAC CCC AAC CTG ACC AAG TTT   609

 V   E   R   F   E   N   L   P   K   I   K   A   Y   M   A   S   D   K    201
GTG GAG AGA TTC GAG AAC CTT CCT AAA ATT AAG GCC TAC ATG GCG TCC GAT AAA   663

 F   M   A   R   P   V   N   N   K   F   A   L   F   K   *                215
TTC ATG GCC AGA CCC GTC AAC AAC AAG TTT GCA TTG TTC AAG TGA tttttaacaaaa  720
tatgttaaataattacactttaacatgtagccataaggacatagaaccctacatacctgtatgtgtacatgt  792
attggagaacaacagttttgtcactctgtatgggtctgtaaaaaaaaattagattctgctattataatatta  864
aacaacacgc[atttg]caaaaaaaaaaaaaa                                           894
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confirming that inductions and inhibitions of GSTs are due to the pollutant.

Results from the pesticide experiments show that the mu class GST was not expressed in either digestive gland or gill in either control and exposed oysters (Figs. 7, 8). In the digestive gland from control oysters, the omega class GST was expressed at a low rate (Figs. 7, 8). Both the omega and pi class GSTs were expressed in gill from control oysters from the two treatments (Figs. 7, 8). During the exposure to both ADI and glyphosate, expression of all GSTs was clearly inhibited in gill tissue (Figs. 7, 8). When 45 cycles of PCR are used, pi, sigma and omega GSTs can be detected on the gel, suggesting that GSTs are strongly inhibited in the gill, but not completely. After 30 days of exposure to ADI, we observed high induction of mRNA synthesis for pi, sigma and omega class GSTs in the digestive gland of oysters (Fig. 7). The omega class GST was expressed in the digestive gland in every sampling from treated oysters from the glyphosate exposure experiment, reaching a maximum value after 21 days of exposure (fivefold compared with control) (Fig. 8). Pi and sigma class GSTs were expressed after 15 days of exposure, followed by inhibition at 21 days and renewed induction after 30 days of exposure (Fig. 8). As for hydrocarbon exposure, no significant variations in GST expression level were observed in control oysters sampled at 7, 14, 21 and 30 days during the two pesticide experiments.

Discussion

In this study, we identified complete mRNA sequences of four genes encoding GSTs in the Pacific oyster *Crassostrea gigas*. These enzymes are known to be involved in phase II biotransformation of xenobiotics. Using specific primers designed from partial sequences obtained in a previous study related to the general

Fig. 4 *Crassostrea gigas*. The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class sigma in oyster (AJ577235). Stop codon is marked by an *asterisk* and untranslated regions are in *lower case letters*. Polyadenylation signals are *underlined* and messenger stability-determining motifs ATTTA and ATTTG are *enclosed in boxes*

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                  M   A   S   Y   R   L   H   Y   F   D   V   R   G   R     14
acactggggataaaar ATG GCC AGC TAC CGA CTT CAC TAC TTC GAC GTT AGG GGC AGG    58

 G   E   I   V   R   M   L   F   K   L   A   Q   A   E   F   G   D   I      32
GGA GAA ATA GTA CGA ATG CTC TTC AAA CTG GCC CAG GCT GAG TTT GGG GAT ATT    112

 R   V   T   Q   G   E   W   T   D   V   K   H   D   T   P   T   G   E      50
CGA GTT ACT CAG GGT GAA TGG ACT GAT GTT AAG CAT GAC ACC CCT ACT GGA GAA    166

 L   P   Y   L   E   V   G   E   K   Q   L   T   Q   S   L   T   I   A      68
CTA CCG TAC TTA GAG GTT GGT GAA AAG CAG CTG ACA CAG AGT CTG ACC ATC GCC    220

 R   Y   L   A   R   E   F   G   L   A   G   D   T   N   W   E   R   A      86
CGC TAC TTG GCC AGG GAG TTC GGT TTA GCT GGG GAC ACG AAC TGG GAG CGC GCT    274

 L   V   E   Q   V   V   D   T   C   D   D   L   R   A   E   N   A   K     104
CTT GTG GAG CAA GTG GTG GAC ACA TGT GAT GAC CTG AGA GCA GAG AAC GCC AAG    328

 I   I   H   E   R   D   P   V   R   L   A   L   M   K   S   K   M   K     122
ATC ATC CAT GAA AGA GAC CCG GTC AGG CTG GCA CTA ATG AAA TCA AAG ATG AAA    382

 D   Q   I   L   P   K   Y   L   N   R   L   T   K   F   L   N   E   H     140
GAC CAA ATA CTT CCC AAA TAC TTG AAC AGA CTT ACT AAA TTT CTA AAT GAA CAT    436

 G   D   R   Y   F   I   G   S   K   I   T   S   A   D   I   A   V   H     158
GGA GAT AGA TAT TTC ATC GGA TCA AAG ATA ACG TCG GCA GAC ATT GCT GTC CAT    490

 E   V   L   T   T   F   L   Q   N   D   P   S   C   L   D   K   H   D     176
GAG GTA TTG ACC ACC TTC CTC CAG AAC GAC CCG TCA TGC CTT GAC AAG CAC GAC    544

 V   L   R   K   H   R   Q   L   V   E   H   H   P   N   L   S   E   Y     194
GTA CTA CGG AAA CAT CGA CAG TTA GTA GAG CAC CAC CCC AAC CTG AGT GAA TAC    598

 L   S   S   R   P   R   F   V   V   *                                     203
CTC TCC TCC AGA CCC CGC TTT GTC GTA TAA ctcgttacctgaatcatcagagccttgcaggc   660
cgtcaatacaggcgatataccgcgatatatcaggggagacaaactgtactgcgcgataccagaggaaaagaa   732
ttaacgtacg[attta]atagtgttactatttttcattcagtataaattaatgtaaggcacgtggtatggttta   804
caaagcgtaaactgtcccctgttatactcgtataacatagaaataatatgattcattccttatactttaatt   876
ttcc[attta]taagtaagaacttatttcctttaatggattttcagaaaacttcaaaatgatgttaaaattgcc   948
atcaaaggcagaaagcgataacagtaaactgtttctcgagaaaaaaaaaaaaaa                    1002
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response of *C. gigas* to hydrocarbon exposure (Boutet et al. 2004), we amplified complete cDNAs encoding the four different GSTs. The molecular masses of the corresponding proteins are in accordance with the mean value of 25 kDa observed in other mollusc species and in other phyla (Fitzpatrick and Sheehan 1993; Blanchette and Singh 1999; Rouimi et al. 2001; Vidal et al. 2002; Guo et al. 2002). The percentage of similarity between *C. gigas* GSTs and those of other species was lower (23–46%) than for the other genes investigated in previous studies of the same species. For example, we obtained 70% homology for the HSP70 gene sequence between *C. gigas* and other species (Boutet et al. 2003). A low degree of homology in GST sequences was also observed in another mollusc species, *Mytilus edulis*, ranging from 32% to 53% (Lüdeking and Köhler 2002). An explanation for this finding might be GST specialisation within an isoform class in addition to selection for substrate specificity in various phyla (Whalen and Boyer 1998). Nevertheless, the homologous nature of stress-gene families and their widespread occurrence among various phyla implies they have fundamental functions in cellular stress responses. The phylogenic tree we constructed also suggests that GSTs evolved from an ancestral gene from which two genes evolved separately to give two distinct clusters of GSTs, mu and pi and omega and sigma respectively. Oyster GSTs constitute distinct branch in the tree because of the lack of GST sequences from other invertebrates, more specifically from molluscs, in the database.

The four GST cDNA sequences presented multiple ATTTA(G) motifs in their untranslated regions. These motifs are known to be correlated with transcript stability (Shaw and Kamen 1986) and have been observed in other cDNAs, such as the sequence encoding glutamine synthetase in the sea urchin *Paracentrotus lividus* (Fucci et al. 1995) and aspartate aminotransferase in rat (Pavé-Preux et al. 1988). The GST omega cDNA sequences characterised from pig and human (Board et al. 2000; Rouimi et al. 2001) and the GST mu cDNA sequence from *Xenopus laevis* (De Luca et al. 2002) did not contain these motifs in their UTRs, while the mu GST

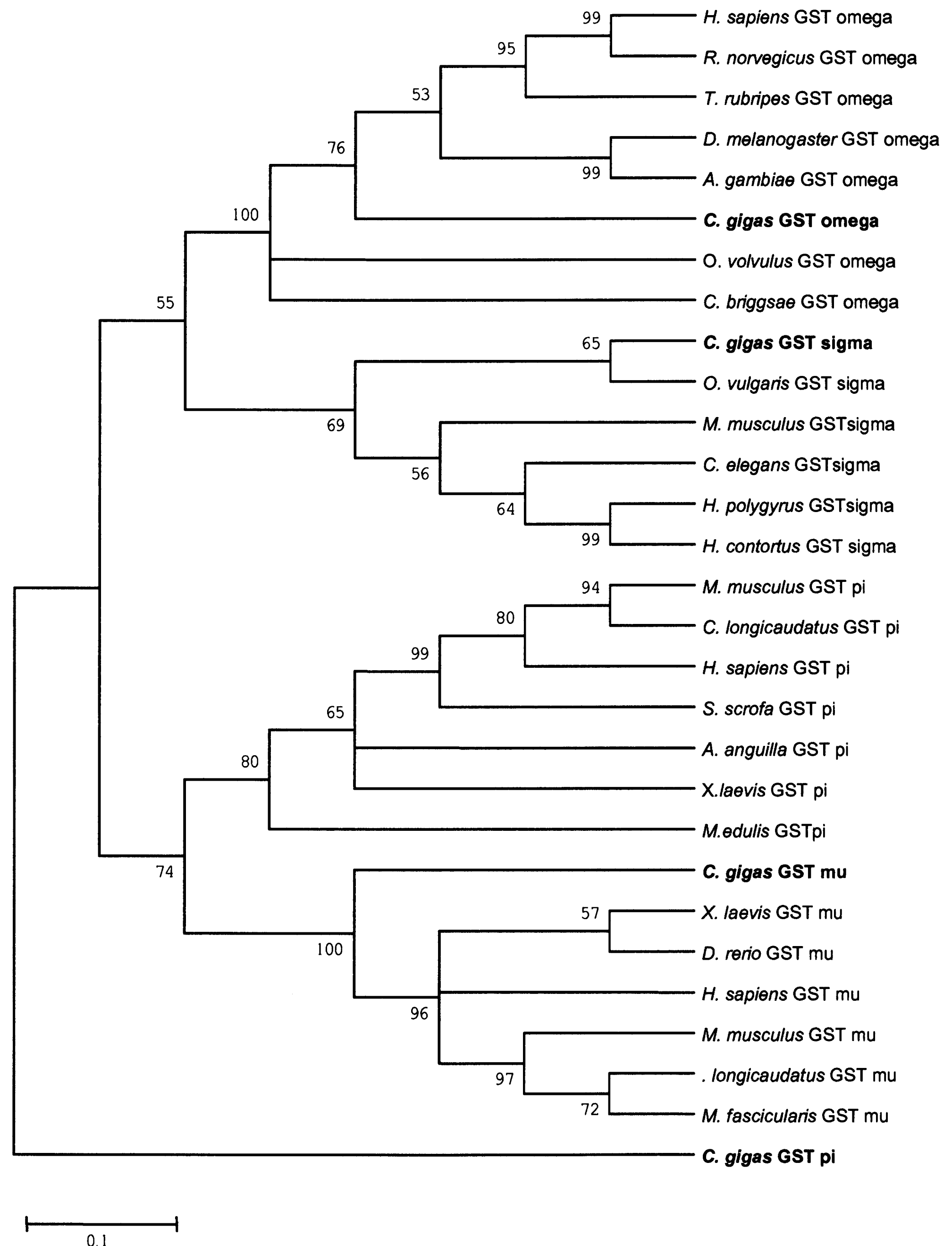

Fig. 5 *Crassostrea gigas*. An unrooted phylogeny showing the most likely relationship between representative GST pi, mu, sigma and omega amino acid sequences. *Branch lengths* are proportional to estimates of evolutionary change. The *number associated with each internal branch* is the local bootstrap probability that is an indicator of confidence. The sequences are *Haemonchus contortus* (AAF81283), *Octopus vulgaris* (P27014), *Caenorhabditis elegans* (NP508625), *Heligmosomoides polygyrus* (AAF36480) and *Mus musculus* (Q9JHF7) for GST sigma; *Mus musculus* (NP861461), *Xenopus laevis* (CAD33920), *Cricetulus longicaudatus* (P46424), *Sus scrofa domestica* (S13780), *Anguilla anguilla* (AAS01601), *Homo sapiens* (AAC13869) and *Loligo opalescens* (AAA97542) for GST pi; *Takifugu rubripes* (AAL08414), *Homo sapiens* (NP004823), *Rattus norvegicus* (XP342063), *Anopheles gambiae* (AAP13482), *Drosophila melanogaster* (NP729388), *Caenorhabditis briggsae* (CAE69580) and *Onchocerca volvulus* (AAF99575) for GST omega; *Xenopus laevis* (AAH54171), *Macaca fascicularis* (AAF08540), *Danio rerio* (NP997841), *Cricetulus longicaudatus* (Q00285), *Homo sapiens* (AAH08790) and *Mus musculus* (NP081040) for GST mu

cDNA sequence from mouse contained two ATTTG motifs in the 3′ UTR (Guo et al. 2002). It is thought that these sites provide targets or recognition sequences for extremely labile endonucleases, thus explaining the effects of inhibitors of protein synthesis to stabilise these mRNAs (Caput et al. 1986; Reeves et al. 1987). The occurrence of these motifs in oyster GSTs suggests that GST transcripts could be more stable and could be converted into functional proteins in the cells at a higher level.

Another interesting 3′ UTR feature was that two polyadenylation signals in the pi class GST cDNA sequence were observed. Multiple polyadenylation sites have already been observed for other genes (Leff et al. 1986). Caizzi et al. (1990) and Smartt et al. (1998, 2001) suggest that the multiple polyadenylation sites indicate the presence of multiple transcripts encoding one protein. Pavé-Preux et al. (1988) found that a single aspartate aminotransferase sequence containing two polyadenylation signals encoded two different mRNAs in rat. They postulated that the two mRNAs resulted from the differential use of these signals during the maturation of pre-mRNA. More analysis will be necessary to determine if the pi class GST sequence encode only one or two different mRNAs.

Analysis of mRNA expression by semi-quantitative, multiplex RT-PCR showed that the four GSTs studied were not expressed in all tissues under normal

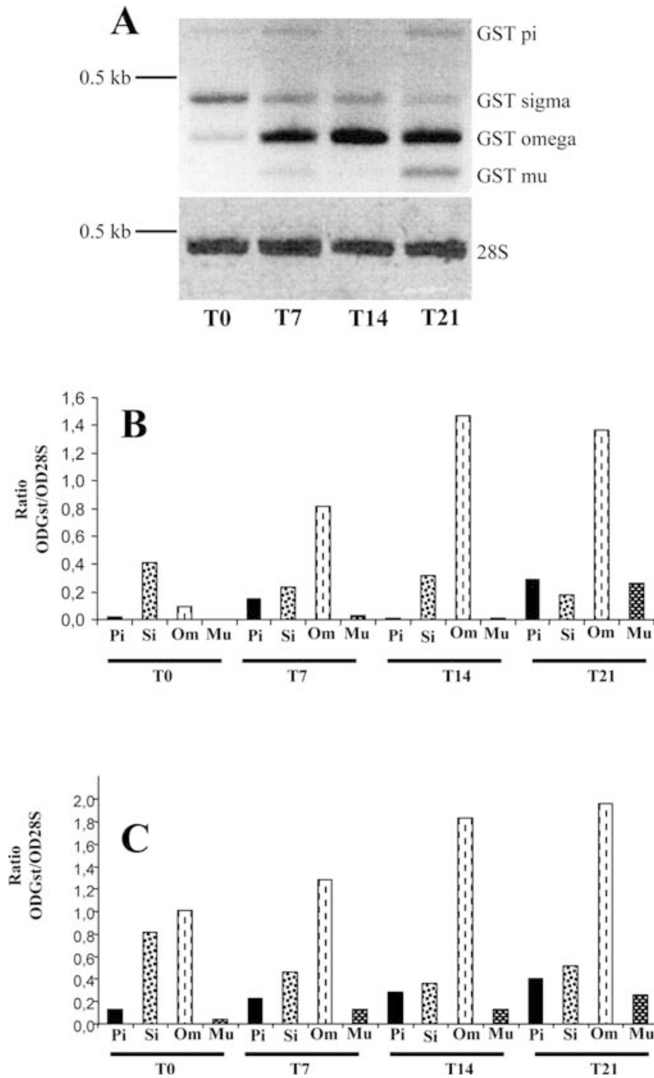

Fig. 6A–C *Crassostrea gigas*. Expression of the four GSTs in the digestive gland of oysters ($n=3$ for each day) exposed to hydrocarbons, using semi-quantitative, multiplex RT-PCR (**A**) (three replicates for each condition). **B**, **C** Values of ratios ODGst/OD28S for the four GSTs at days 0, 7, 14 and 21 (*T0–T21*) in digestive gland (**B**) and gill (**C**)

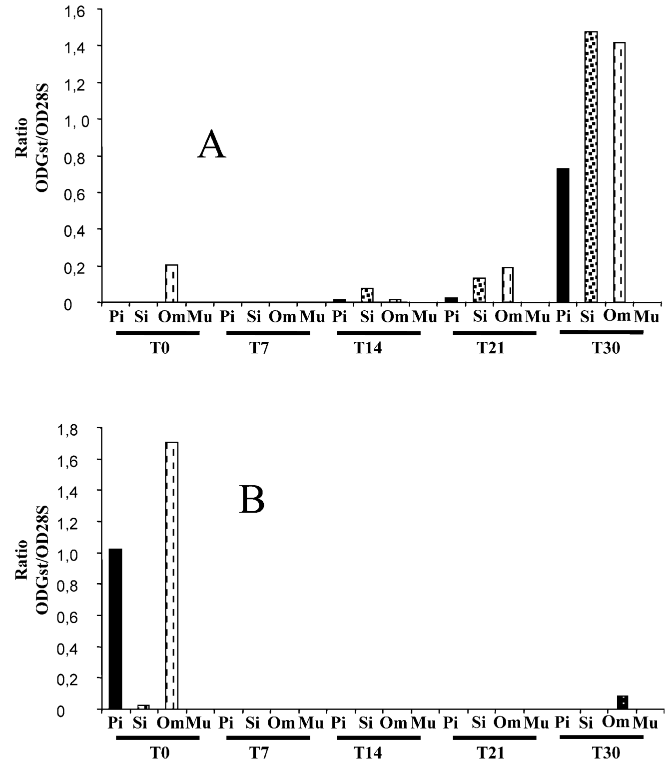

Fig. 7A, B *Crassostrea gigas*. Expression of GSTs in digestive gland (**A**) and gills (**B**) of oysters ($n=3$ for each day) exposed to ADI, using semi-quantitative, multiplex RT-PCR (three replicates for each condition). Expression is presented as the ratios ODGst/OD28S for the four GSTs at days 0, 7, 14, 21 and 30

conditions. In the present report, we observed that only the omega class GST was expressed at a low rate in both the gill and digestive gland from the control oysters, whereas the mu class GST was absent in those tissues from the control oysters. In addition to tissue-specific differences, the relative abundance of the various RNAs could vary under different physiological conditions. The control oysters from the hydrocarbon and pesticide experiments did not express the same GST classes. Control oysters from the hydrocarbon experiment expressed pi, sigma and omega class GSTs, while control oysters from the pesticide experiments expressed only omega and pi class GSTs. It has been demonstrated that GSTs are associated with cell proliferation (Terrier et al. 1990; Lüdeking and Köhler 2002) and that the intracellular level of these enzymes may be co-ordinated by other genes in response to oxidative stress (Salinas and Wong 1999). Perhaps because cell proliferation in molluscs is dependent on their physiological condition, expression of GST classes varied in the control oysters used for the two experiments. Studies on GST activities in barnacle showed that maximal GST activities were detected in the summer period, followed by a gradual decrease between July and October, to reach a minimum in the winter period (Niyogi et al. 2001). In our studies, GSTs omega and pi present a higher mRNA concentration in the gills of samples collected in September for the pesticide experiments than in those collected in December for the hydrocarbon experiments. Moreover, results obtained with ADI and glyphosate also varied, particularly in the digestive gland, leading to a more complicated interpretation of the possible biological role of GST in pesticide detoxification. If glyphosate seems to induce GST mRNA expression, an opposite effect is observed for the cocktail atrazine, diuron and isoproturon. GST omega was expressed in both control and treated oysters, in both tissues studied. The expression of omega class GST was observed in several tissues in human and pig (Board et al. 2000; Rouimi et al. 2001). Members of the omega class have been reported to be involved in radiation resistance in lymphoma cells (Kodym et al. 1999) and in protection against oxidative stress (Board et al. 2000; Dulhunty et al. 2001).

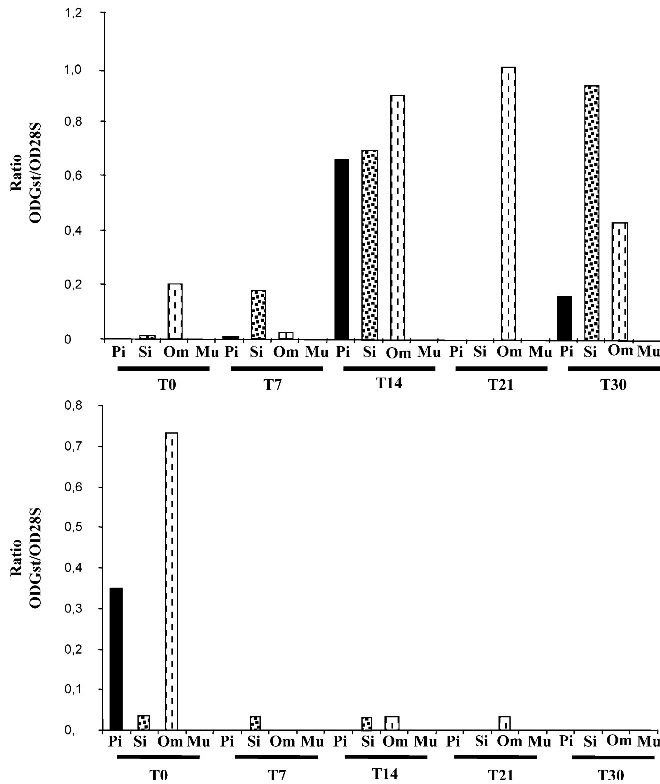

Fig. 8A, B *Crassostrea gigas*. Expression of GSTs in digestive gland (**A**) and gills (**B**) of oysters ($n = 3$ for each day) exposed to glyphosate, using semi-quantitative, multiplex RT-PCR (three replicates for each condition). Expression is presented as the ratios ODGst/OD28S for the four GSTs at days 0, 7, 14, 21 and 30

Following an oxidative stress, a number of cellular proteins form S-thiol adducts with glutathione and cysteine (Hanson et al. 1999). The formation of these adducts can inactivate the enzymatic functions of affected polypeptides (Ravichandran et al. 1994; Jahngen-Hodge et al. 1997). Omega class GSTs may reduce this type of S-thiol adduct and restore enzymatic function (Board et al. 2000).

Based on the pesticide exposure results, three of the four GSTs displayed an increase in their expression level in the digestive glands of oysters exposed to both glyphosate and ADI, while the mu class GST does not seem to be expressed. Previously, it has been demonstrated that isoproturon causes a marked induction of GST in rat liver (Schoket and Vincze 1985; Hazarika and Sarkar 2001) and that both atrazine and isoproturon generate strong selection in *C. gigas* populations (Moraga and Tanguy 2000). The toxicity of these two pesticides caused a mortality rate of 60–70% in *C. gigas* populations at concentrations of 0.1 and 0.2 mg l^{-1} after 2 months of exposure. Conversely, comparable exposure to diuron does not cause mortality (Moraga and Tanguy 2000). Other studies report decreases in GST activity in response to some pesticides such as in the mussel *Anodonta cygnea* (Robillard et al. 2003) or the rat (Hazarika et al. 2003). But little clear information on the molecular processes involved in GST regulation by pesticides is available, particularly for bivalves, and results concerning induction or inhibition of GST by pesticides and insecticides vary according to the species studied. Moreover, pi and sigma class GSTs do not seem to be expressed in the digestive glands of control oysters. This differential expression between challenged and control oysters, especially for pi and sigma class GSTs in the digestive gland, may be useful as a marker for pesticide exposure. Mu class GST appears to be weakly expressed in oysters, in both gill and digestive gland, and is only induced in the presence of hydrocarbons. Mu and omega class GST mRNA expression may be similarly useful as a biomarker for hydrocarbon exposure.

To summarise, we characterised, for the first time, four cDNAs encoding GSTs in the oyster *C. gigas*. Their sequence data formed the basis of an expression study that used semi-quantitative, multiplex RT-PCR methods to follow the simultaneous expression of the four GSTs in the same sample. The results showed tissue-specific, time- and treatment-dependent differential expression of the GSTs in oysters. Furthermore, omega and mu class GST mRNA expression may be useful as a marker of hydrocarbon exposure, and pi and sigma class GST mRNA expression may be useful as a marker of pesticide exposure in monitoring programmes. Nevertheless, an analysis of GST expression patterns will be conducted over a year to study the effect of organ-specific patterns and seasonal variations on the four GSTs in oyster. The influence of other stress effects, such as heavy metals or abiotic parameters (hypoxia), would also present fruitful lines of further investigation.

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